Chemokine receptor CXCR4 and CXCR7 as a Novel Target Against Small Cell Lung Cancer

Research Thesis

Presented in partial fulfillment of the requirements for graduation with research distinction in the undergraduate colleges of The Ohio State University

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June 2012

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Abstract

Small cell lung cancer (SCLC) is a highly aggressive malignancy with a limited spectrum of therapeutic options. We are planning to analyze chemokine receptor 4 (CXCR4) and 7 (CXCR7) as promising and emerging biomarkers for SCLC. Both CXCR4 and CXCR7 have been shown to be highly expressed in various types of cancers. It has also been suggested that CXCR4 expression may be increased in patients with metastatic disease. Recently, enrichment of CXCR4+ stem-like sub-population of cells was observed in tumors treated with cisplatin, suggesting that these populations may be resistant to chemotherapies. The main objective of this study is to determine expression and clinical utility of CXCR4/CXCR7 as novel biomarkers and therapeutic targets for inhibiting SCLC growth and metastasis.

The central hypothesis of this study is that chemokine receptors CXCR4 and CXCR7 could be used as novel biomarkers for assessing the prognosis and serve as therapeutic targets in treatment of small cell lung carcinoma (SCLC). In addition, we hypothesize that the differential CXCR4/CXCR7 expression in the cytomembrane or nucleus can be associated with prognosis and disease-free survival of SCLC patients. We also hypothesize that these receptors could be used as novel targets to inhibit SCLC growth and metastasis, as antagonists of these receptors have been shown to inhibit growth and metastasis of other types of cancers. Furthermore, we hypothesize that CXCR4/CXCR7 inhibition in highly-tumorigenic SCLC stem-like cells may be an attractive strategy to overcome chemo-resistance and relapse in SCLC patients. Therefore, analysis of these receptors may provide a sound rationale for pursuing novel inhibitors of CXCR4/CXCR7 in clinical trials for SCLC patients.

The main objective of this study is to determine the expression and clinical utility of CXCR4 and CXCR7 as novel biomarkers and therapeutic targets for inhibiting SCLC growth and metastasis.

Introduction

BACKGROUND: Small cell lung cancer (SCLC) comprises 15-20% of lung cancer and is a highly aggressive malignancy which usually presents at the advanced stage due to early metastasis. Not many therapies are available for SCLC and its 5-year overall survival is only 5%. Furthermore, SCLC is characterized by high relapse rates and poor prognosis due to drug resistance. Therefore, it is important to develop novel therapies for improving poor prognosis of SCLC. An emerging target for cancer therapy is CXCL12 also known as stromal-derived factor-1 alpha (SDF-1α), which has been shown to bind to its cognate receptors CXCR4 and CXCR7[1,2]. Recently, CXCR4 and CXCR7 have been shown to be highly expressed in various tumors, including non small cell lung cancer (NSCLC) [3,4]. CXCL12, upon binding to CXCR4 or CXCR7, stimulates various signaling pathways related to cell survival, proliferation and migration. CXCR4 overexpression is also correlated to poor clinical outcomes and survival in various cancer including breast, prostate. In addition, CXCR4/CXCL12 signaling axis plays an important role in organ selective metastasis as CXCR4 overexpressing cancer cells have been shown to metastasize to organs such as bone, lymph nodes, liver, lung, and brain, which produce high amounts of CXCL12 and thus provide a favorable microenvironment [5]. There are some reports which suggest correlation between CXCR4 and clinical outcomes in non small cell lung cancer (NSCLC) [6,7]. It has been suggested that CXCR4 expression may be increased in patients with metastatic disease. CXCR4 expression has also been shown to be both in cytoplasm

and nucleus; however, there are conflicting reports regarding the clinical relevance of such differential expression [6,7]. It has also been shown that nuclear expression of CXCR4 in NSCLC tumor cells significantly correlates to lymph node metastasis. It has been shown that high expression levels of CXCR4 are correlated with metastasis to brain in NSCLC that have shorter survival rates. CXCR7 expression has also been observed in various tumors, including breast, prostate, pancreatic and NSCLC [4,8]. However, not much is known about the expression of CXCR7 and CXCR4 in SCLC patients samples. Present study will further analyze the expression of CXCR4 and CXCR7 in tissue microarray (TMAs) derived from neuroendocrine patients samples.

Lung Cancer

Lung cancer is a Disease characterized by uncontrolled growth of cell and tissue located in the lungs. It is the leading cause of cancer-related death worldwide with about 85% of cases being related to cigarette smoking. About 15% of people have never smoked that develop lung cancer. This may due to genetic mutations in the epidermal growth factor gene (EGFR). It is theorized, though not clearly established, that environmental association could be a cause, such as radon gas, a breakdown of product of naturally occurring radium and uranium, exposure to secondhand smoke and exposure to carcinogens, such as asbestos, radiation, arsenic, chromates, nickel, chloromethyl ethers, mustard gas, or coke-oven emissions. It can spread rapidly beyond the lung through a process called metastasis. Most cancers which begin in the lung are carcinomas derived from epithelial cells. The main types are small cell lung cancinoma (SCLC) and non-small cell lung carcinoma (NSCLC). The diagnosis is typically made by a chest x-ray or Computer tomography (CT) scan and confirmed through biopsy. But, depending on the stage of

the disease, treatment includes surgery, chemotherapy, radiation therapy, or a combination of the three. The prognosis still remains poor, despite medical advances in treatment, with 15% of patients surviving greater than 5 years from the time of diagnosis. The 5 year overall survival rate is <1% for patients with stage IV (metastatic) disease. Survival can be improved through focusing attention on smoking cessation, early detection, and research into the genetic profile of lung tumors and developing novel forms of therapy. Symptoms can include cough, chest discomfort, chest pain, weight loss, and hemoptysis, though many patients with metastatic disease are without clinical symptoms [9].

Small Cell Lung Cancer

SCLC is a high aggressive, rapidly growing cancer. It is the most aggressive form of lung cancer, with approximately 60% of patients have widespread metastatic disease upon the time of diagnosis, which is one reason for the high fatality rate [9,10]. It spreads much more quickly than non-small cell lung cancer. Typically starts in the breathing tubes (bronchi) in the center of the chest. Although the cancer cells are small, the grow very quickly and create large tumors which typically metastasize to other parts of the body, such as the brain, liver, and bone. About 15% of all lung cancer cases are SCLC, and is slightly more common in men than women. Almost all cases of SCLC are due to cigarette smoking, and it is rare in people who have never smoked.

There are two different types of SCLC, Small cell carcinoma (oat cell cancer) and combined small cell carcinoma, with oat cell being the most common. SCLC can be classified as limited or extensive. Limited is when the cancer is only in the chest and can be treated with radiation

therapy, where extensive is when the cancer has spread outside the chest. SCLC has often already spread to the brain with no symptoms or other signs. Patients with smaller cancers may receive radiation therapy to the brain called prophylactic cranial irradiation (PCI). Surgery helps very few patients with SCLC because the disease has often spread by the time it is diagnosed and chemotherapy or radiation therapy is still be needed. The survivability depends on how much the lung cancer has spread. The spread of the cancer and side effects of surgery, chemotherapy, and/or radiation therapy make this a very complicated and deadly disease [10].

SCLC tends to metastasize to the marrow, where NSCLC tend to metastasize to the osseous tissue. The marrow microenvironment has a high constitutive CXCL12 expression. CXCR4 activation induces migratory and invasive responses, as well as adhesion to marrow stromal cells in a CXCR4 and integrin-dependent manner in SCLC cells. The signaling via CXCR4 on SCLC cells also induces activation and signaling of tumor-associated integrins. The adhesion to the marrow stromal cells or extracellular matrix proteins by integrins can protect the SCLC from chemotherapy induced apoptosis, conferring drug resistance and tumor cell growth. This can be inhibited via CXCR4 antagonists. This shows the CXCR4 antagonist seems to interfere with survival signals from the microenvironment [11].

CXCR4/CXCL12 Signaling

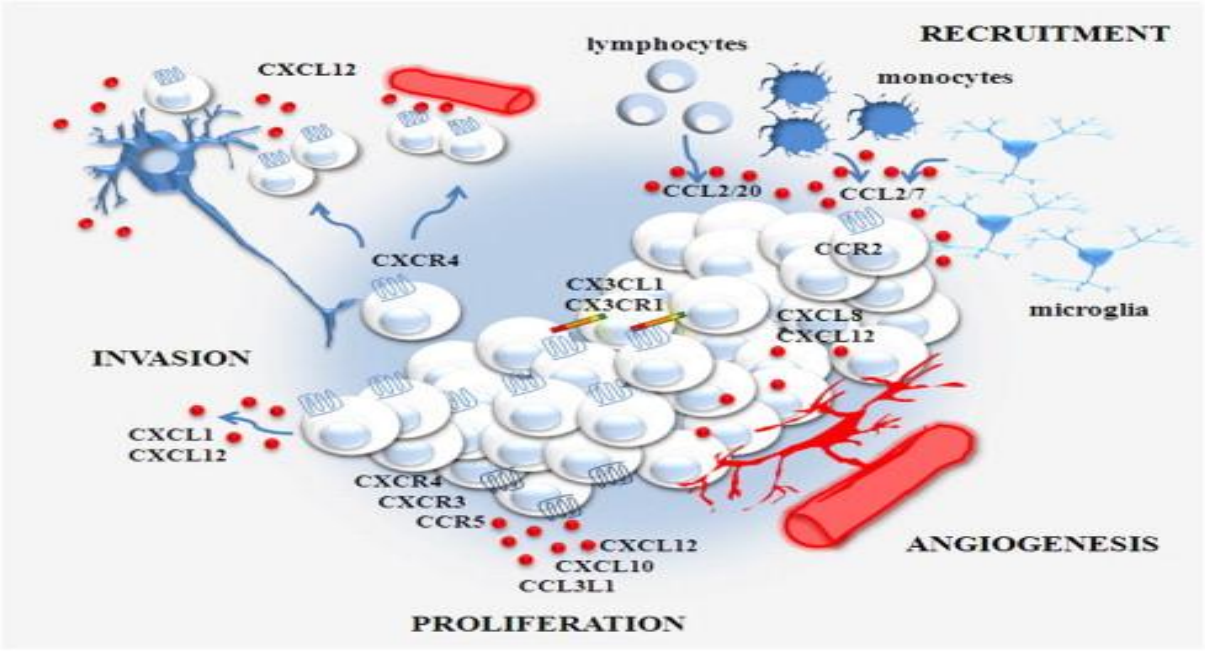


Fig.1 CXCR4/CXCL12 mediated functions. The CXCR4/CXCL12 axis has a role in regulating trafficking of various types of cells, including hematopoietic and progenitor cells. It has roles in Roles in: recruitment (chemotaxis of stromal cells), angiogenesis (new vascularization), proliferation (multiplication of cells), and invasion/migration (metastasis) [12].

CXCR4 is a chemokine receptor type 4, also known as fusin or CD184. It is alpha-chemokine receptor specific for SDF-1, a molecule endowed with potent chemotactic activity for lymphocyte [13]. It is located on the cell surface, and acts with CD4 protein to support HIV entry into cells. It also is expressed on multiple cell types including: lymphocytes, hematopoietic stem cells, endothelial and epithelial cells, and cancer cells. CXCL12 transduces a signal by increasing intracellular calcium ion levels and enhancing MAPK1/MAPK3 activation. CXCR4 also induces intracellular signaling through several divergent pathways initiating signals related: chemotaxis, cell survival/proliferation, an increase in intracellular calcium, and gene transcription [14].

CXCR4/CXCL12 axis has been shown to enhance tumor growth and metastasis by modulating tumor stroma through activation of cancer-associated fibroblasts (CAFs) and recruitment of $CXCR4^+$ endothelial precursor cells, thereby, enhancing angiogenesis. In addition, CXCL12 has also been shown to increase angiogenesis through endocrine mechanism by recruiting $CXCR4^+$ endothelial pre-cursor cells to the tumor stroma. CXCL12 is involved in recruitment of various CXCR4+ bone-marrow derived cells (BMDC), including CD11b+ myelomonocytes (that differentiate into macrophages) and endothelial precursor cells that stimulate blood vessel formation [15]. CXCR4/CXCL12 signaling has also been shown to enhance angiogenesis through upregulation of VEGF and IL-8. CXCL12/CXCR4 axis may also stimulate tumor growth by enhancing recruitment of $Gr1^+CD11b^+$ myeloid derived suppressor cells (MDSCs) to the tumor stroma [16]. MDSCs have been shown to enhance tumor growth by inhibiting tumor immune response.

Recent studies indicate that cancer stem cells are resistant to chemotherapy and therefore play an important role in tumor progression, metastasis and development of drug resistance in various tumors. These cancer stem cells are thought to be resistant to chemotherapeutic drugs since these drugs only target dividing cells. Cancer stem cells only represent a subpopulation of cells in a growing tumor but are capable of initiating tumor growth and metastasis. CXCR4 expression is found on normal stem cells of several organs and tissues [17] and since many tumor cells express CXCR4, it is highly possible that malignant cancer cells are derived from CXCR4-expressing normal tissue stem cells. Recent data demonstrates that glioma tumor stem-like cells promote tumor angiogenesis and vasculogenesis via a CXCL12/CXCR4 pathway [18-20]. It has been shown that CXCR4 is predominantly overexpressed in progenitor cells compared to differentiated tumor cells and that CXCL12 stimulated proliferation of glioblastoma-derived

progenitor cells but not in differentiated glioblastoma cells. Thus, these results suggest that the CXCL12/CXCR4 interaction mediates glioma progenitor/stem cell proliferation. Mesenchymal stem cells (MSC) have been shown to play an important role in the development of ovarian cancer metastasis and resistance to treatments. Recently, it was shown that tumor associated mesenchymal stem cells protect ovarian cancer cells from hyperthermia through CXCL12/CXCR4 signaling.

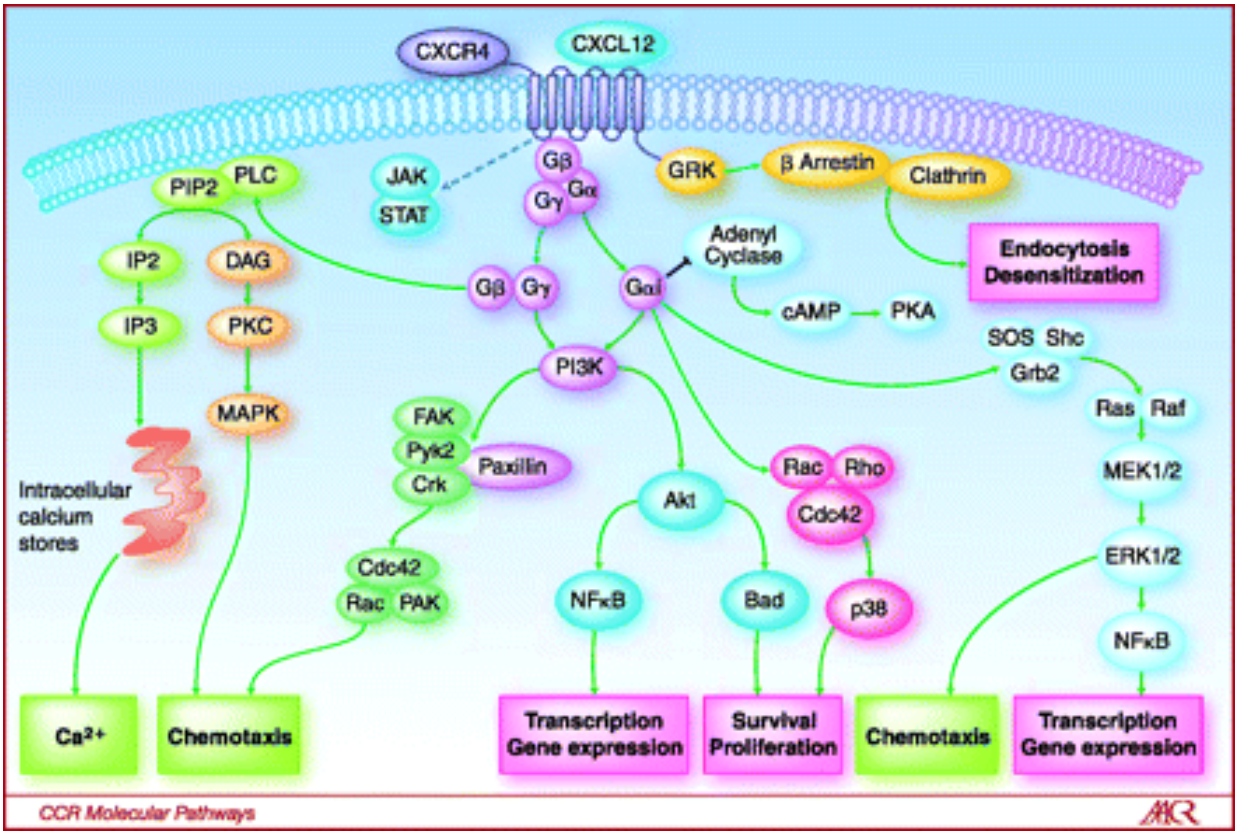


Fig.2 CXCR4/CXCL12 axis signaling pathway. CXCL12 activates CXCR4 signaling through Akt (involved in survival and proliferation), ERK1/2 (involved in chemotaxis and gene expression), FAK (involved in chemotaxis), and STAT3 (involved in chemotaxis, proinflammation, tumor growth). (103)

CXCR4 has also been shown to be expressed in highly-tumorigenic stem-like lung cancer cells derived from NSCLC. Furthermore, an enrichment of $CD133^{+}$/CXCR4 population was observed

in tumors treated with cisplatin, suggesting the existence of chemo-resistant $CXCR4^+$ stem-like subpopulations which have high tumorigenic and metastatic properties [21]. In addition, CXCL12, the ligand of CXCR4, has been shown to enhance proliferation of stem-like lung cancer cells. In this study, we will determine if the CXCR4/CXCL12 pathway play an important role in regulating proliferation of cancer stem cells derived from SCLC tumors.

A series of targets and therapeutic strategies for the treatment of SCLC are currently being investigated. However, all patients ultimately develop resistance against these agents, including chemotherapy. Recently activation of chemokine receptors CXCR4 and CXCR7 through its ligand CXCL12, have been shown to enhance tumor resistance to various therapies by promoting tumor cell survival, invasion, and enhance survival of tumor-initiating cell phenotypes or cancer stem cells (CSC), as well as facilitate metastasis [21]. CAFs have also been shown to promote the proliferation of breast cancer $CD44^+CD24^-$ stem cells through their ability to secrete CXCL12 [20]. Therefore, potential use of anti-CXCR4 and CXCR7 agents may sensitize tumors to currently available therapies by blocking CXCL12/CXCR4 and CXCL12/CXCR7 pathways.

There are various agents that have been developed that block CXCL12/CXCR4 and CXCL12/CXCR7 functional activity. One of these agents, AMD3100, also known as plerixafor, is an FDA approved drug with a relatively mild toxicity profile [22]. CXCR7 specific inhibitors, CCX733 and CCX2066, have also been generated. Chemotherapy drugs or an anti-angiogenic agent in combination with CTCE-9908 have been shown to possess enhanced anti-tumorigenic and metastatic effects. Currently, CTCE-9908 is undergoing Phase 1 and Phase II clinical trials in hepatocellular carcinoma [22]. Recently, CXCL12/CXCR4 inhibitors used in combination with other anti-cancer treatments or therapies have shown promising efficacy [8]. The reason could be that CXCL12/CXCR4 is as an important mechanism for development of drug resistance.

Recent clinical studies have shown that circulating CXCL12 plasma levels are significantly enhanced in recurrent glioblastoma (GBM) patients after treatment with anti-VEGFR agents. Circulating plasma CXCL12 have also been shown to be increased in rectal cancer patients after treatment with anti-VEGF treatments with chemoradiation. Furthermore, the enhanced CXCL12 levels are associated with higher levels of metastatic disease. Recently it has been suggested that in multiple tumor types, stimulation of CXCR4/CXCL12 plays an important role in development of drug-resistance and enhancement of metastasis [8]. Most of the studies have been done the regarding CXCR4/CXCL12 pathway, however not much is known about CXCL12/CXCR7, which is emerging as an important pathway in regulating the growth of solid tumors. We are planning to develop preclinical models to closely recapitulate the clinical features and response to chemotherapies, especially cisplatin, in SCLC patients. We will analyze the effect of CXCR4 and CXCR7 in blocking SCLC growth *in vivo*. In addition, we will determine if CXCR4 inhibitors inhibit proliferation of stem-like cancer cells and sensitize SCLC tumors to chemotherapeutic agents, especially cisplatin. Mechanistic understanding of CXCL12/CXCR4 and CXCL12/CXCR7 in SCLC should provide novel insights about using anti-CXCR4 and CXCR7 drugs for developing improved treatments against SCLC. Thus analyzing expression of CXCR4/CXCR7 in patient samples, as well as *in vivo* mouse model studies using CXCR4/CXCR7 inhibitors, will be critical for pursuing CXCL12 pathway as a novel target against SCLC, as well as enhancing efficacy of chemotherapy. Thus, targeting CXCL12/CXCR4 signaling axis will prevent the development and maintenance of tumor promoting cancer stem cells and therefore, may prove to be a useful antitumor therapeutic strategy in the future.

Chemokimes and Chemokine receptors

Chemokines are a family of small, chemotactic cytokines. Cytokines are proteins secreted by cells. They have the ability to induce directed chemotaxis in nearby responsive cells. Some chemokines are proinflammatory and are induced under an immune response. They recruit cells of the immune system to a site of infection. Other chemokines are homeostatic and are involved in controlling the migration of cells during tissue maintenance or development. They exert their effects by interacting with G-protein-coupled, transmembrane receptors found on the surface of their target cells. Chemokines act as a chemoattractant, guiding the migration of cells to a target location containing an increased chemokine concentration (the source of the chemokine). They can also direct lymphocytes, white blood cells, to lymph nodes to screen for invasion of pathogens, illicit an immune response, or promote wound healing. Some chemokines promote angiogenesis, the growth of new blood cells. They can also be used to guide cells to tissues that provide critical signals for cellular maturation. Chemokine release is often stimulated by pro-inflammatory cytokines, such as interleukin 1 [23].

Proteins are classified as chemokines due to their structural characteristics. They are small molecules, approximates 8-10 kilodaltons in size with the presence of four cysteine residues in conserved locations which are key to forming their teriatry structure. There are four kinds of chemokines: CC, CXC, C, and CX_3C chemokines. They are labeled due to their varying spacing of the first two cysteines. CC chemokines have two adjacent cysteines near the animo terminus. There are 27 of them found to date. These chemokines typically possess four cysteines, but are known to possess up to six, including CCL1, 15, 21, 23, and 28. CC chemokines induce migration of monocytes, NK cells, and dendritic cells. CXC chemokines, which I'm studing, have cysteines separated by one amino acid. There have been 17 different CXC chemokines

found to date, which are subdivided into two subgroups. The subgroups are divided into groups with and without a specific amino acid sequence of glutamic acid leucie-arginine immediately before the cysteine of the CXC motif. The ones with induce the migration of neutrophils, interacting with receptors CXCR1 and 2. The ones without tend to be chemoattractant for lymphocytes, or carcinogenic cells in my case. There are seven CXC chemokine receptors discovered thus far. The C chemokines are distinct in having only two cysteines total, with one near the amino terminus. There are two C chemokines, XCL1 and 2. These chemokines attract T cell precursors to the thymus. The fourth type of chemokine is the CX_3C chemokine, possessing three amino acids between the first two cysteines. There has only been one discovered, CX_3CL1. It is both secreted and tethered to the surface of the secreting cell, making it both a chemoattractant and adhesion molecule [24].

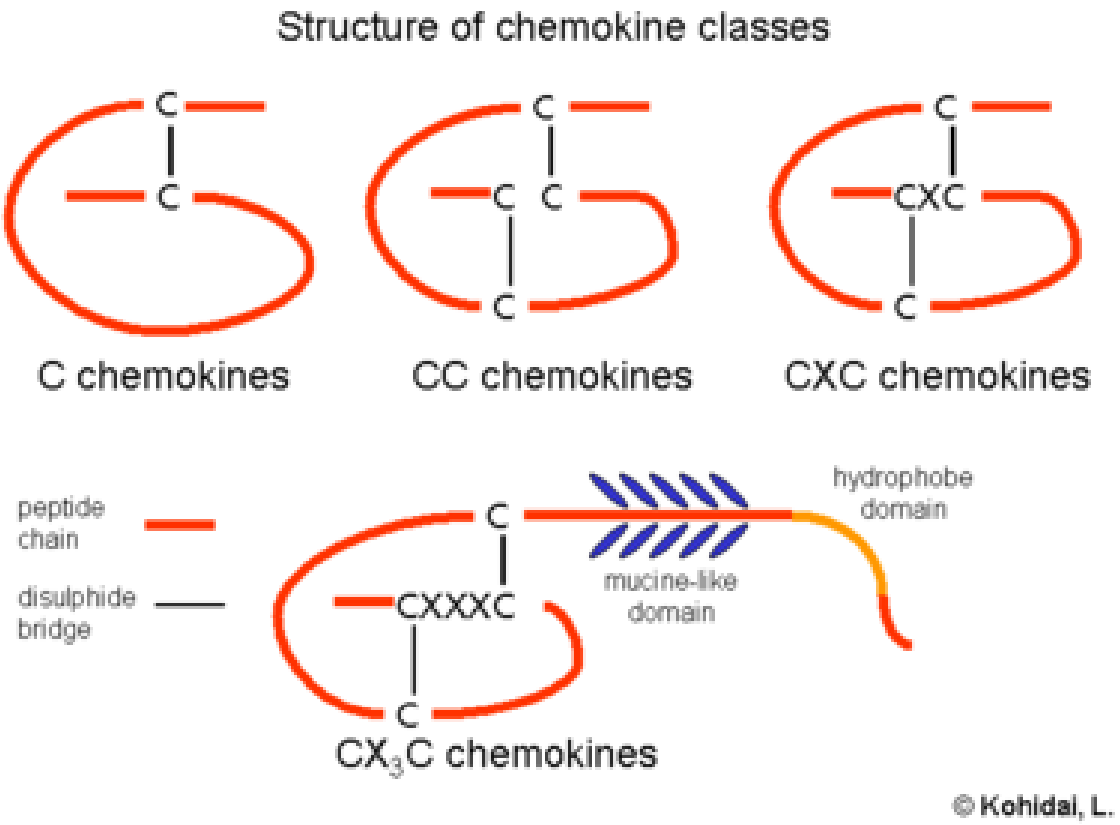


Fig. 3 Structure of chemokine classes. Chemokines are proteins classified by their structural characteristics. They have four cysteine residues in conserved locations which are key to forming tertiary structure. There are four kinds: C, CC, CXC, and CX_3C, which are labeled due to varying spacing of first two cysteines [25].

Chemokine receptors are G protein-coupled, transmembrane proteins. They tend to be found on leukocytes and recently on carcinogenic cells. There is about 19 different chemokine receptors, divided into four families relating to the chemokine ligand they bind. The receptors tend to share many structural features: similar size, acidic N-terminal end, seven helical transmembrane domains with three intracellular and extracellular loops, and an intracellular c-terminus. The first two extracellular loops of the receptor has a conserved cysteine residue, allowing formation of a disulfide bridge between the loops. The G-proteins are coupled to the C-terminal end to

allow intracellular signaling after activation. The N-terminal domain contains the ligand binding specificity [26].

CXCL12 is the chemokine stromal cell derived factor-1 (SDF-1). Tumor cells from patients with SCLC tend to express high levels of functional CXCR4 receptors for the chemokine CXCL12. It's a homeostatic chemokine that is constitutively secreted by marrow stromal cells, playing a key role for homing of hematopoietic cells to the marrow. Tumor cells from patients with SCLC tend to express high levels of functional CXCR4 receptors for the chemokine CXCL12 . Tumor cells often metastasize to the bone marrow because of this [27]. CXCR4 is the cognate receptor for CXCL12, which is unique. CXCR4 promotes tumor progression by both direct and indirect mechanisms, making it a novel target for therapeutic agents [11].

CXCL12, a chemokine, and CXCR4, its cognate receptor, have been shown to play roles in favoring tumor growth, progression, and immune escape through numerous mechanisms. CXCL12 and CXCR4 constitute a chemokine-receptor axis, known to be expressed in a variety of tumors with a strong correlation to poor clinical outcome. The CXCL12 and CXCR4 expression has been detected in many cancers, and CXCR4 antagonists were originally developed for treatment of AIDS [11].

CXCL12 and CXCR4 gene-deleted mice possessed a lethal phenotype, indicating the monogamous relationship between the receptor and its chemokine. The mice suffered from deficient B-lymphopoiesis, myelopoiesis, and abnormal neuronal and cardiovascular development. This unique selectivity may be necessary to maintain HSCs in the metaopoietic microenvironment and marrow-specific homing of circulating HSCs. Recent studies have shown that CXCR4 antagonists can affect mobilization of HSCs [11].

Inhibitors of CXCR4/CXCL12 Signaling Axis

The ability to block migration and dissemination of tumor cell metastasis, tumor growth, survival signals, and pro-angiogenesis effects show that CXCR4/CXCL12 has the potential to be a therapeutic target. There are several types of CXCR4/CXCL12 antagonists being used to inhibit this axis, such as bicyclams (AMD3100), T22, TN14003, CTCE-9908, and ALX40-4C, which are analogs and peptides based on the amino-terminal region of the chemokine, CXCR4. The specific antagonist of CXCL12 binding to CXCR4, AMD3100, inhibits the CXCL12-mediated calcium mobilization, chemotaxis, and GTP binding. It does not cross react with other chemokine receptors. AMD3100 antagonists are macrocyclic polyamines which have been shown to inhibit the X4 strain of HIV binding to CXCCR4, which acts a as a co-receptor for HIV. Through functional studies, it has been shown that strong and direction interaction occurs between CXCR4 and bicyclams without receptor internalization. AMD3100 has been shown to inhibit CXCR4/CXCL12 mediated tumor growth and metastasis in both *in vitro* and *in vivo* studies in various cancers [28].

CXCR4 antagonists, such as AMD3100, have been shown to sensitize SCLC cells to cytotoxic drugs, such as etoposide. This suggests that the CXCR4/CXCL12 signaling axis can be used as a novel target in drug-resistant SCLC therapy. It is predicted that combining CXCR4 antagonist with cytotoxic chemotherapy may be an attractive strategy to overcome chemo-resistance and relapse in lung cancer. In other studies, where another CXCR4 inhibitor, TN14003, which is derived from 14-mer peptide antagonists of CXCR4 (T140) has been used, including *in vivo* models for breast cancer it has been shown that in addition to limiting metastasis of breast cancer via inhibiting migration. This shows that TN14003 may also be useful as a diagnostic

tool to identify CXCR4 receptor positive tumor cells in culture and tumor samples. TN14003 has also been shown to be effective in other cancers, including small cell lung cancer (SCLC) malignant melanoma, and pancreatic cancer [28]

CXCR7 Signaling

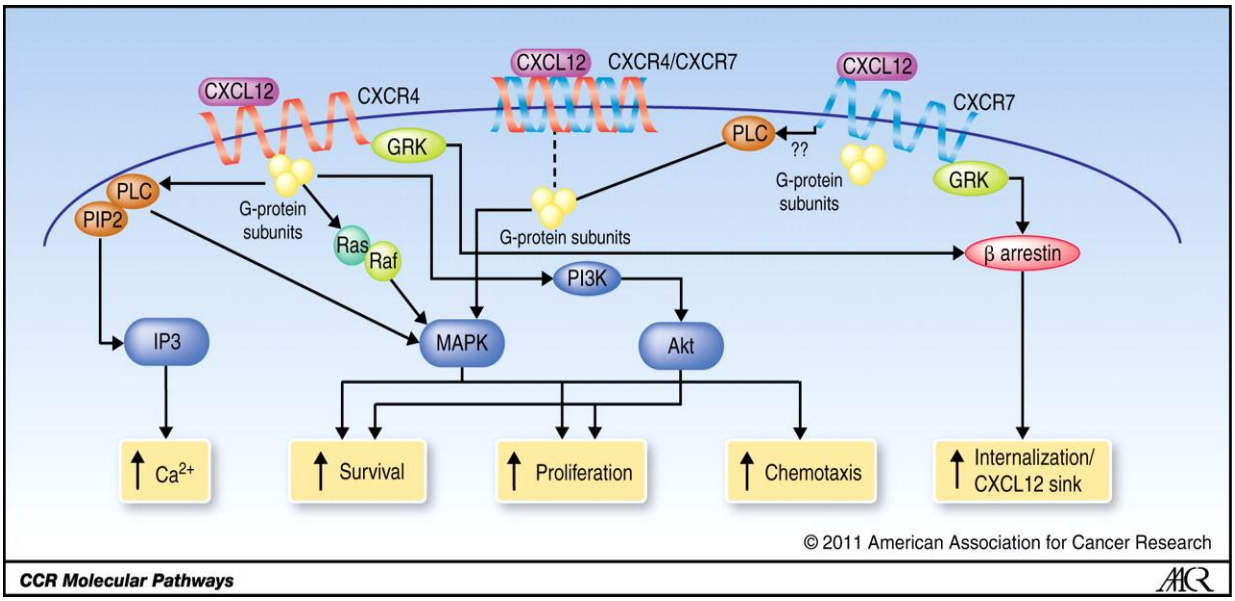


Fig 4 CXCL12/CXCR4/CXCR7 Pathway. Activation of CXCR7 by CXCL12 increases the CXCL12-induced signalling of CXCR4. CXCR4 leads to increased survival, proliferation, and chemotaxis [29].

Recently, it has been found that CXCR4 and CXCL12 are not a monogamous pair, but that CXCL12 also binds to CXCR7. In addition, CXCR7 may also bind to CXCL11. But, CXCR7 didn't induce signaling, such as when CXCL12 binds to CXCR4. It seems to form functional heterodimers with CXCR4 and enhance CXCL12-induced signaling. There seems to be a specialized role for CXCR7 in endothelial biology and valve development. CXCR7 is expressed

in a wide range of tissues in humans and are up-regulated in some tumors, similar to CXCR4. Similar to CXCR4, CXCR7 can facilitate angiogenesis, and its blockage, as well as CXCR4's blockage can lead to inhibition of tumor growth in several mouse models [30].

CXCR7 doesn't seem to function as a classical chemokine receptor with a lack of detection of calcium flux or migratory behavior of CXCR7 expressing cells after stimulation with CXCL12 or CXCL11. It may actually behave as a decoy or chemo-kine transporting receptor, similar to other endothial-expressed receptors. An alternate explanation is that it may not function alone, but modulate CXCR4 functions through the formation of heterodimeric complexes. These chemokine receptor heterodimerizations increase the sensitivity and dynamic range of the response. There was a stronger Ca^{2+} flux in cells coexpressing CXCR4 and CXCR7 than in CXCR4 only containing cells. It was concluded that CXCR4/CXCR7 form heterodimers, and coexpression of these two receptors induces a stronger Ca^{2+} flux than CXCR4 alone and modulates downstream signaling through ERK1/2 [31].

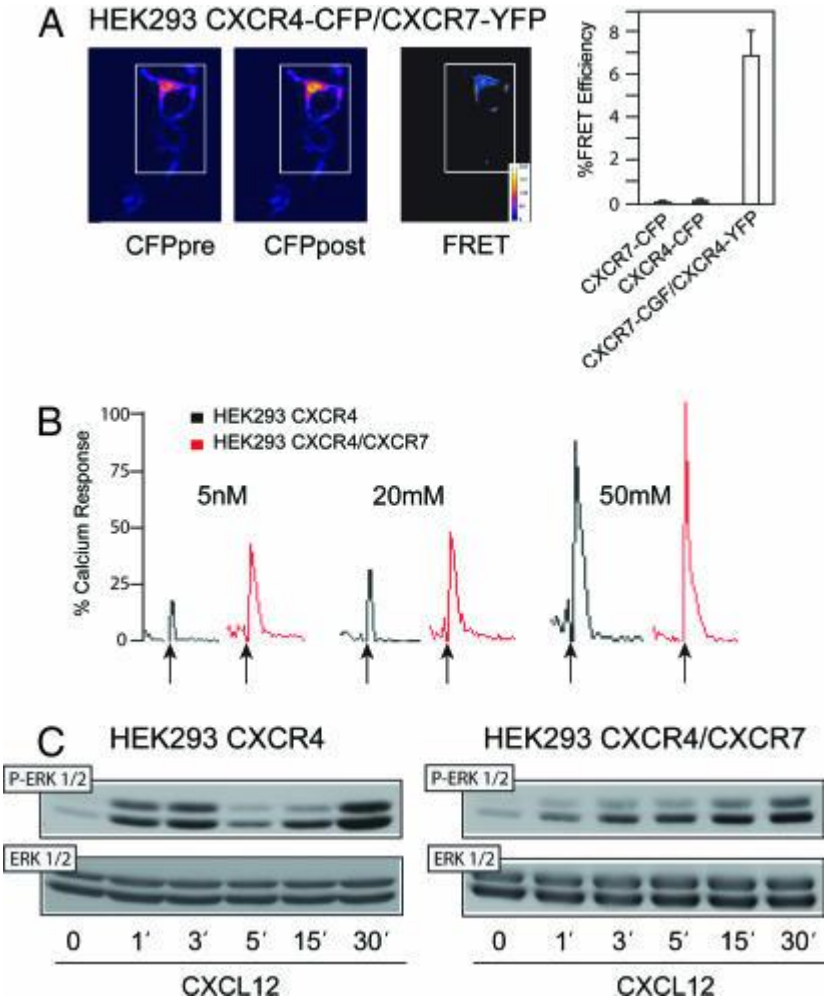


Fig. 5 FRET Measurements of CXCR4 and CXCR7: FRET measurements were used to show that preformed dimers of CXCR4 and CXCR7 were detected at the cell membrane in the absesnse of ligand and with the evidence of there being intracellular heterodimer pools [31].

Signal Transduction

Cell signals are transmitted using G-proteins after ligand binding. The activation of G-proteins activates the enzyme, phospholipase C (PLC). PLC then cleaves a molecule called phosphatidylinositol (4,5)-bisphosphate (PIP2) into two second messenger molecules known as Inositol triphosphate (IP3) and diacylglycerol (DAG), which trigger other intracellular signaling

events. DAG can activate another enzyme, protein kinase C (PKC), and IP3 triggers the release of calcium from intracellular stores, which can be measured for chemokine receptor activation. Many signaling cascade events can result, generating responses, such as chemotaxis, degranulation, and release of superoxide anions, change in the avidity of integrins. Transcription gene expression, and survival proliferation [32].

Stromal Cell

Stromal cells are connective tissue cells of any organ, which support the function of the parenchymal cells [mutant cells in a cancer of a single lineage] of that organ. Examples include: fibroblasts, immune cells, pericytes endothelial cells, and imflammatory cells. Interactions between stromal cells and tumor cells tend to play major roles in cancer growth and progression [33]. Stromal cells in the tumor microenvironment at primary or metastatic sites can regulate tumor progression. Adhesion of the cancer cells to the stromal cells supports the growth of the neoplastic cells from the high level of CXCL12 expression and integrins, which can activate CXCR4 and/o growth promoting tyrosine kinases.

CXCR4 and CXCL12 pathway

Manipulating, blocking, or impairing the CXCR4/CXCL12 pathway may impact tumor pathogenesis, including immune dysregulation [34]. Reverse transcriptase-polymerase chain reaction and flow cytometry have demonstrated CXCR4 mRNA and CXCR4 surface expression in SCLC cell lines. Immunohistochemistry of primary tumor samples from SCLC patients have also revealed high expression of CXCR4. CXCL12 sparked CXCR4 endocytosis, actin polymerization, and a high activation of phosphor-p44/42 mitogen-activated protein kinase in

SCLC cells. CXCL12 has also been shown to induce SCLC cell invasion into extracellular matrix and firm adhesion to marrow stromal cells. The CXCL12 released by the stromal cells attracts cancer cells containing the cognate receptor, CXCR4. CXCL12 binds to the G-protein-coupled receptor, CXCR4, aka CD184 [27].

The CXCR4/CXCL12 pathway is involved in leukocyte, white blood cells, trafficking or the directing of the immune system. Tumor metastasis is not a random process, as it shares many similarities with leukocyte trafficking. Chemokines play a key role in trafficking and homing of hematopoietic cells, all bone-marrow-derived cell types in the blood. These cells are uniquely selective in their migratory response to CXCL12. This regulates the specific homing of HSC in the marrow microenvironment. This also results in the dissemination and marrow homing of malignant hematopoietic and nonhematopoietic cells [27]. CXCL12 helps retain B-cell precursors in close contact with protective stromal cells in the hematopoietic microenvironment, where its expression is essential for normal B-cell development. CXCL12 supports the survival or growth of a variety of normal or malignant cell types, including hematopoietic progenitors, germ cells, leukemia B cells, and breast carcinoma cells. The CXCR4/CXCL12 axis also aids in tissue repair and regeneration using selective recruitment of circulating or resident progenitor cells. Hypoxia-inducible factor-1 (HIF-1) induces CXCL12 expression in ishemic areas, recruiting repair cells to tissue damage. HIF-1 plays an important role in generating gradients of CXCL12 in the marrow microenvironment. HIF-1 also enhances the expression and function of CXCR4 on normal and malignant cells [11].

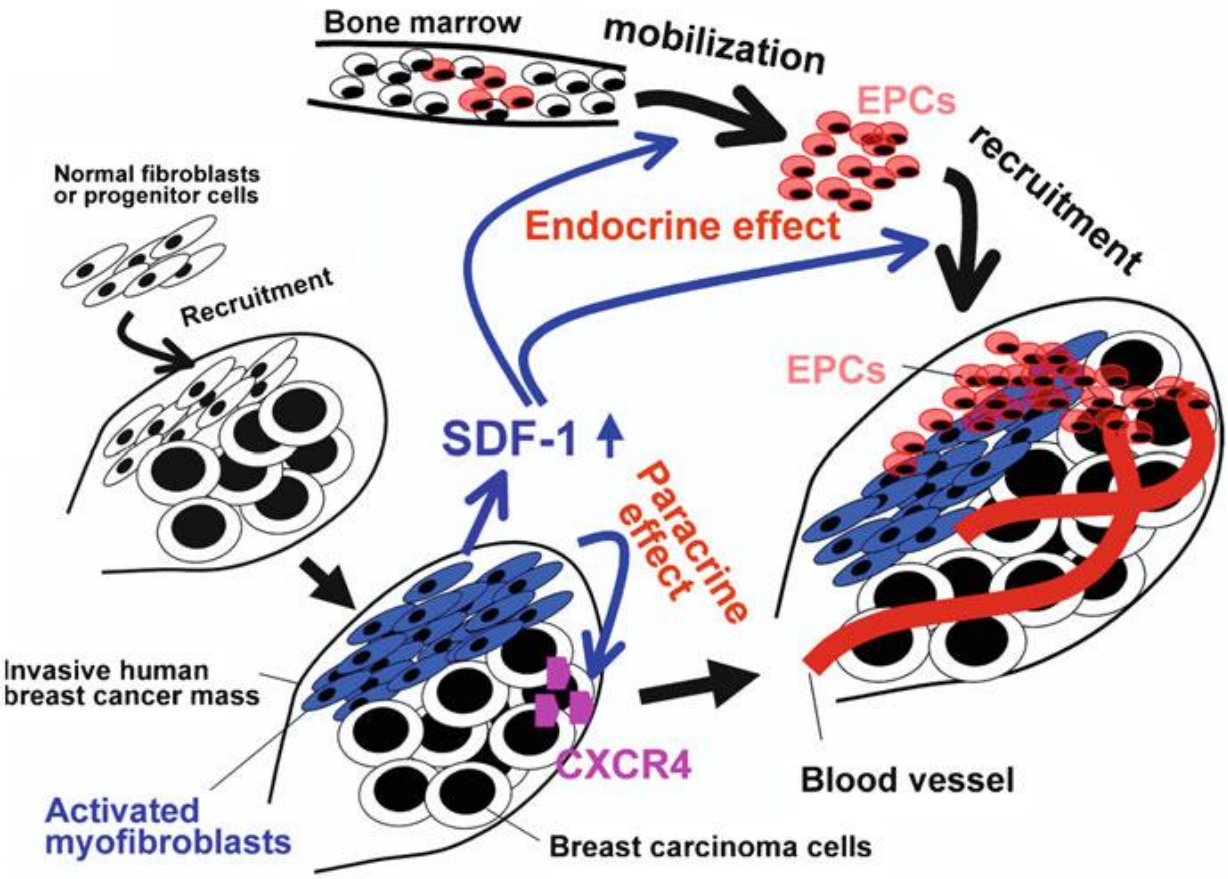


Fig.6 CXCL12 Recruitment of Stromal Fibroblasts. The CXCR4/CXCL12 signaling pathway regulates tumor growth and metastasis in cancers through modulating tumor stroma (an active element of the tumor microenvironment), tumor cells, fibroblasts, endothelial cells, immune cells, and extracellular matrix (ECM). CXCR4/CXCL12 signaling axis accelerates cancer growth/metastasis through the increased development of carcinoma-associated fibroblasts (CAFs) [35].

CXCL12 can stimulate cell proliferation in cancers by direct activation of kinases, such as kinase B and mitogen-activated protein kinase, and direct protection of cancer cells from apoptosis. Tumor tissues and the surrounding stroma can stimulate VEGF-mediated angiogenesis and the recruitment of endothelial progenitor cells from the bone marrow. The CXCl12 released can also

mediate tumor invasion and metastasis by impairing cell adhesion to integrins, attracting CXCR4 cancer cells, and increasing matrix metalloproteinase expression [34].

integrins are receptors that mediate the attachment between the cell and the tissues surrounding it, including other cells or the extra cellular matrix. They play a role in cell signaling and can regulate cell shape, motility, and cell cycle. These receptors contribute to both the inside-out and outside-in signaling. They therefore relay information about the cell to the environment and information about the environment to the cell [36].

CXCL12 has also been shown to repel tumor specific effector T cells (Teff) and recruit suppressive cell populations at tumor sites. ThIs includes interleukin-10 producing plasmacytoid dendritic cells, regulatory T cells (Treg), and myeloid-derived suppressor cells. It was demonstrated that CXCR4 chemokine receptors can induce integrin activation on SCLC cells [27]. Integrin activation results in the arrest, firm adhesion, and transendothelial migration into tissues where chemokine gradients direct the localization and retention of the cells [11]. SCLC cells express various α and β integrin chains that allow for interactions with the tumor microenvironment. This results in enhanced tumorigenicity and resistance to chemotherapeutic agents due to β1 integrin-stimulated tyrosine kinase activation. SCLC cells express functional CXCR4 which plays a role in adhesive interactions with stromal cells [27]. CXCR4 is essential for metastatic spread of cancer cells to organs where CXCL12 is expressed. This allows tumor cells to access cellular chices that favor tumor survival and growth, such as the marrow. Stromal derived CXCL12 can, itself, stimulate survival and growth of neoplastic cells in a paracrine fashion, promoting tumor progression. It can also promote tumor angiogenesis by attracting endothelial cells to the tumor microenvironment, further promoting growth and longevity of the tumor cells [11].

Tumorgenic cells which take advantage of the CXCR4/CXCL12 axis tend to metastasize to the skeletal system, including both the bone marrow and the osseous tissue. SCLC tends to metastasize to the bone marrow, where other lung cancer types tend to metastasize to the osseous tissue. This is an important negative prognostic factor in SCLC [11].

Methods and Materials

Cells

The SCLC cell lines of NCI-H69 and NCI-H345 were obtained from xxx, the NSCLC cell line A549 was obtained from xxx, and Jurkat cell lines were obtained from xxx. The NCI-H69 and Jurkat cell lines were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% Penicillin-streptomycin-glutamine. The NCI-H345 cell lines were maintained in HITES serum free medium, consisting of DMEM/Ham's F-12 medium, 1:1 mix with 1% insulin 0.005 mg/ml Transferrin beta-estradiol 10 nM HEPES 10 mM L-glutamine 2 mM solution and 1% Penicillin-streptomycin-glutamine. The A549 cell lines were maintained in DMEM medium containing 10% fetal calf serum (FCS) and 1% Penicillin-streptomycin-glutamine. The cells were kept in a humidified incubator containing an atmosphere of 5% CO_2 and 95% air at 37 ^{o}C. The cells had their media replaced every 2-3 days through collection by centrifugation and dispersion into fresh media, changing their container about every 7 days. Attached suspension cells were detached by shaking flask gently if needed. The A549 cells were briefly rinsed with 0.25% trypsin- 0.53 EDTA solution, then about 2 ml of the solution was added to the container and placed in the incubator for 5 minutes. The cells were collected, spun down, then added to fresh media in a new container.

Antagonist

The specific chemokine receptor antagonist, AMD3100, was dissolved in sterile PBS as a 5 mg ml^{-1} stock solution and diluted appropriately with sterile PBS in a new microcentrifuge tube for experiments of 500 or 1000 ng ml^{-1}.

CXCL12

The specific chemokine ligand CXCL12, SDF-1, was dissolved in distilled H_2O to make a stock solution of 100 ng/ul and diluted with the culture medium to 200 ng/ml for experiments.

RIPA

The RIPA solution was made using 50 mM Tris HCL pH8, 150 mM NaCl, 1% Np-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecylsulfate (SDS).

FACS analysis

For the surface staining of CXCR4 and CXCR7, NCI-H69, NCI-H345, A549, and Jurkat cells were grown to suspend $2x10^6$ cells per 100 ul of the respective primary antibody. NCI-H69, NCI-H345, A549, and Jurkat cells were harvested from their respective flask, washed, and resuspended in ice cold washing buffer (PBS containing 0.5% FBS). The cells were then incubated in 100 ul of the appropriate primary antibody. The cells were allowed to sit in the primary CXCR4, CXCR7, and appropriate primary IgG isotype control antibody for 60 minutes

at 4 ^{o}C. The Cells were washed thrice with FACS buffer and resupsended in 100 ul of secondary antibody solution for 30 minutes. The cells were washed thrice with FACS buffer and added to cold 1% paraformaldehyde solution. The Fluorescence was quantified using a BD flow cytometer [37,38].

Western Blot

NCI-H69, NCI-H345 and A549 cells were lysed in RIPA lysis buffer (*50 mM Tris HCL pH8, 150 mM NaCl, 1% Np-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecylsulfate (SDS).* 50 µg of proteins was loaded on 4–12% SDS–polyacrylamide gels (Invitrogen) under reducing conditions, transferred to nitrocellulose membranes (BioRad) and blocked with 5% milk. Membranes were incubated overnight with primary antibody, washed three times, and incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibody. The membranes then were washed and stained using a chemiluminescence system (ECL-Pierce) and exposed to X-ray film (Kodak). *Anti-CXCR4, anti-GAPDH, anti-pERK, anti-tERK, anti-p-STAT3, anti-p-Akt, anti-FAK, and anti-FOX03A primary antibodies were used with their coordinating anti-mouse or anti-rabbit secondary solution.*

Signaling

SDF-1 was used to determine the level of protein expression over time intervals ranging from 0 to 30 minutes. The NCI-H69 cells were harvested to 10^6 cells per 200 ul of 1x PBS. 200 ul of the cell solution was distributed to a microcentrifuge tube with enough SDF-1 being added to the

rest of the cell solution to make a 200 ng/ml SDF-1 solution. The cell solution was distributed to microcentrifuge tubes containing 200 ul of the cell solution and placed in the incubator for 0, 1, 3, 5, 7, 10, 15, and 30 minutes at 37 ^{o}C with 5% CO_2, 95% air, respectively. 1 ml of cold PBS was added to each microcentrifuge tube after the target time and placed on ice. The proteins were extracted and western blots run as described above. This was repeated at 0, 1, 3, 5, 7, 10, and 15 minutes for NCI-H59.

Signaling with the addition of CXCR4 antagonist AMD3100

The signaling was reproduced for both NCI-H69 and NCI-H345 at 0, 5, and 10 minutes with varying amounts of CXCR4 antagonist AMD3100 of 0, 500, or 1000 ng ml^{-1} for the NCI-H345 cells and 0, 10, and 20 μM concentrations for the NCI-H69 cells. The samples were pretreated with AMD3100 for 1 hour in an incubator at 37 ^{o}C with 5% CO_2, 95% air, respectively, then treated with 200 ng ml^{-1} SDF-1 for the targeted times. Cold 1x PBS was added to the samples after the targeted times had elapsed and the samples placed on ice until protein extraction. The proteins were extracted and run on western blots as described above.

Chemotaxis and chemoinvasion assays

H69 Cell lines were assessed for migration utilizing a chemotaxis assay. Several studies with differing chamber pore filter sizes of 3, 5, 8, and 12 μm were used, with the Jurkat cell line used as a control in some instances. A solution of 600 ul of serum free RPMI medium containing differing amounts of CXCL12 was added to the lower chamber. A 150 ul (500 ul for the 12 um

pore sized chambers) cell solution of a 10^6 cells ml^{-1} was washed and suspended in serum free RPMI medium in the top chambers and placed in the incubator for a targeted time. The chambers were coated with nothing, fibronectin, or collagen. The fibronectin (fibronectin in 1x PBS) and collagen (1:3 collagen to 70% EtOH) were added to the bottom of the top chamber and allowed to dry overnight. Each varying amount of SDF-1 containing chamber was completed in duplicates. After the targeted time was met, the number of cells were counted in both the bottom and top chambers, then the coated filters were stained and the SCLC cell invasion was quantified.

The first migration assay consisted of 2 ug ml^{-1} fibronectin treated chambers containing pore filters of 8 um and 0 and 100 ng ml^{-1} SDF-1 in the bottom chamber for the Jurkat cells and 0, 50, 100, and 200 ng ml^{-1} SDF-1 in the bottom chamber for the NCI-H69 cells. After three hours, the bottom chamber was checked for cell migration. The number of Jurkat cells in the bottom chamber were counted at 24 hours and the H69 cells at 48 hours. The top chambers weren't dyed and discarded.

The Second migration was split into two sides with one side containing wells with fibronectin and the other side without. Only NCI-H69 cells were used with chambers having pore filters of 12 μm. Serum free medium containing 0, 100, and 200 ng ml^{-1} was used in the bottom chambers for both sides of the migration assay.

The third migration involved 8 um pore sized chambers using 0, 100, and 200 ng ml^{-1} SDF-1 with cells washed with 1 ml of trypsin + EDTA before addition to the chambers. One side was counted after 24 hours and the other after 48 hours.

The fourth attempt at migration involved 8 um pore sized chambers over 48 hours involving NCI-H69 cells. The bottom chamber contained serum free RPMI medium of 0, 200, and 400 ng

ml^{-1} SDF-1. One side contained no membrane while the other contained a collagen membrane. The cells were washed with 1 ml of trypsin + EDTA in the incubator before begin loaded into the top chamber.

The Fifth migration assay used a 5 um pore sized chamber coated in collagen with the cell clusters physically separated using a 1000 ul pipetman. The cells were placed in a solution of RPMI medium with 0.25% BSA and an insulin/transferrin/sodium selenite solution a day before, which was also used as the medium during migration. The chambers were stained after 24 and 48 hours.

Results

Morphology of NCI-H69 cell line

The NCI-H69 cell line grows in bunches which were hard to separate. EDTA alone typically didn't separate them, but trypsin + EDTA would, though usually cause fatalities. This mixture will damage the cell and typically kill the cells several days later, even if removed quickly after administration. The cells grow quickly, up to about 10 million in about two weeks if taken care of well. They are extremely small and circular. They suck the nutrients from the medium quickly, changing its rosy orange color to a yellow in typically less than 4 days in a ratio of 10^6 cells ml^{-1} medium. They are suspension cells. The cells tend to die after 4 days of neglect.

Morphology of NCI-H345 cell line

The NCI-H345 cell line grows much slower than NCI-H69, though it is also a SCLC cell line. These cells are also circular and similar in size. They grow in clusters, yet break apart with gentle shaking of their container. They are also suspension cells. The cells tend to die after 6 days of neglect and change the red color of their HITES' medium slowly to an orange hue. The cells are heavy and tend to settle at the bottom of their flask, sometimes sticking to the sides.

Morphology of A549 cell line

A549 cell line is an adherent, non small cell lung cancer (NSCLC) cell line. They grow extremely fast and have triangular, elastic looking shapes. They gain about 80% confluence of 8 million cells in about 8 days. Their CXCR4 expression failed in comparison to the NCI-H69 and NCI-H345 cell lines.

Expression of CXCR4 in SCLC cells

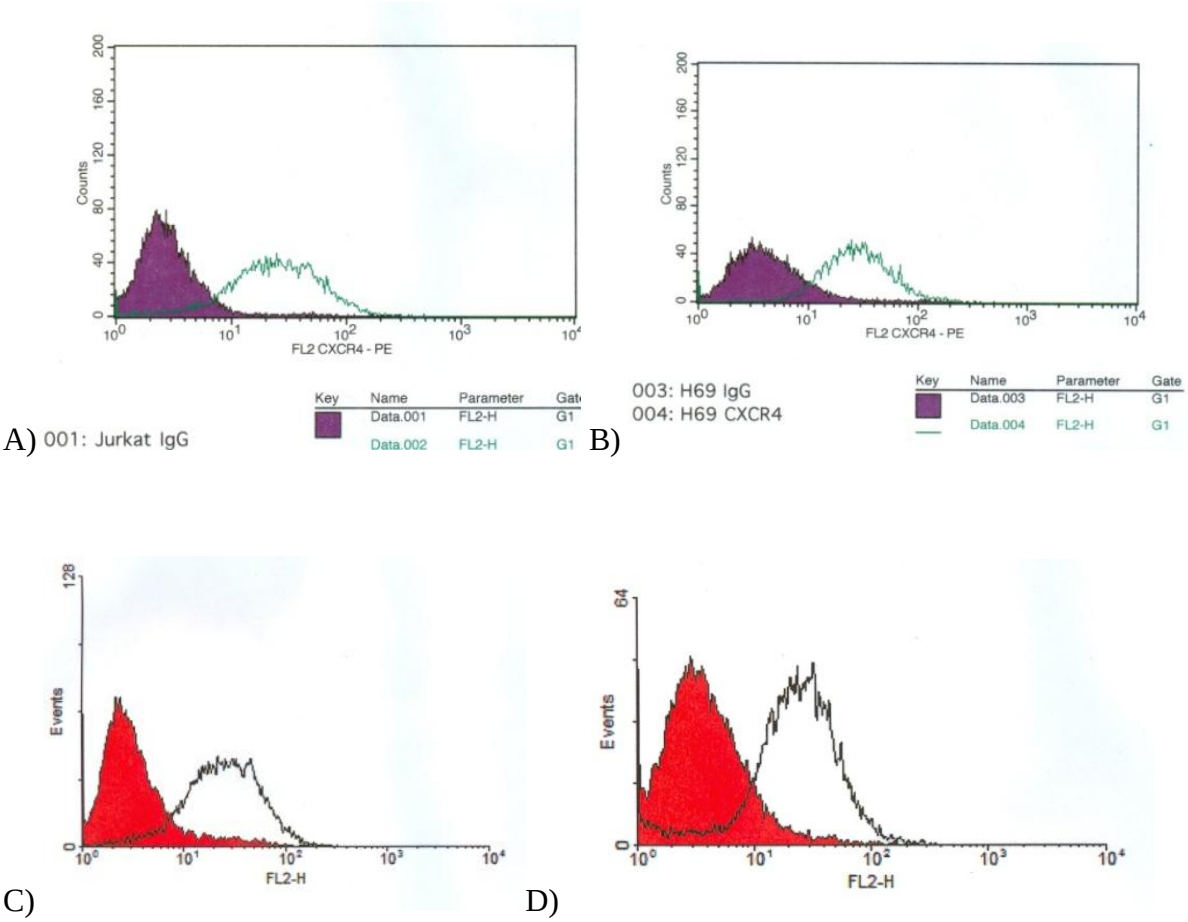


Fig.8 Expression of CXCR4 in NCI-H69 Cell Line. A/C) Jurkat cells (control). B/D) NCI-H69 cells. The Jurkat cell line is known to have a high CXCR4 protein expression and was used as a positive control. The difference in areas under the dark peak and white peak show the high level of CXCR4 protein expression. The experiment was repeated twice showing the answer to be precise and reproducible. FACS analysis has shown there is a large amount of CXCR4 expression in NCI-H69 cell lines. The large amount of surface area under the green graph on the left and the black graph on the right exemplify this.

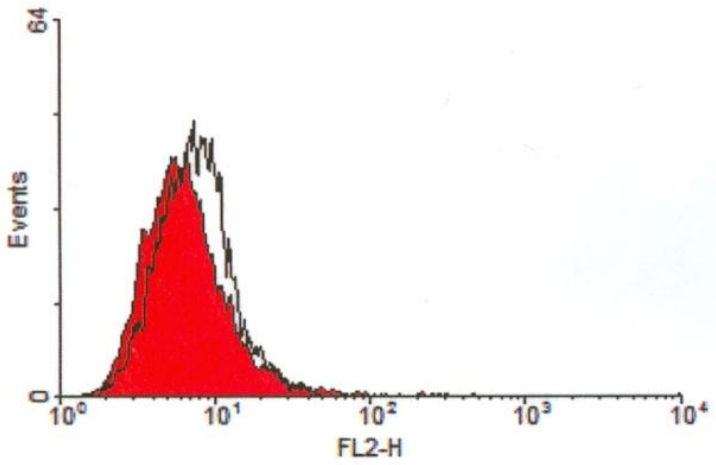


Fig. 9 Expression of CXCR4 in A549 Cell Line. Expression of CXCR4 in A549 cells through FACs analysis. The small gap between peaks shows a low expression in the A549 cell line.

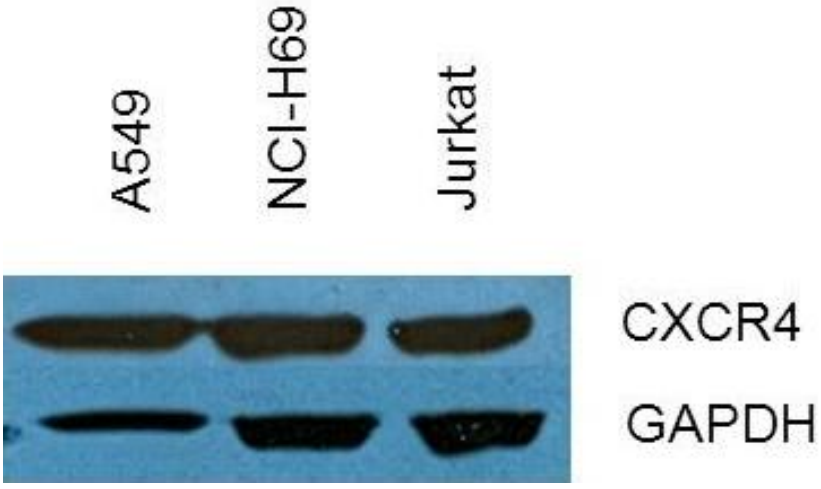


Fig. 10 Expression of CXCR4 in NCI-H69 and A549 Cell Lines Through Western Blot. There is expression of the CXCR4 protein in both the A549 and NCI-H69 cell lines. The Jurkat cell line was used a control. There was nearly equal amounts of protein shown by the consistency of density in GAPDH expression.

Expression of CXCR7 in NCI-H69 Cell Line

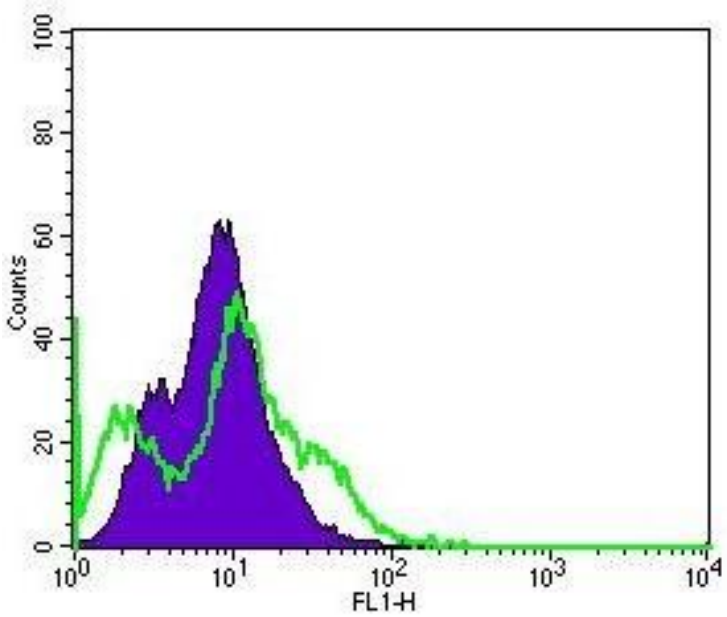


Fig. 11 Expression of CXCR7 in NCI-H69 Cell Line. This graph shows the expression of CXCR7 in the NCI-H69 cell line. There is a 30% expression in the NCI-H69 cell line.

Western Blot

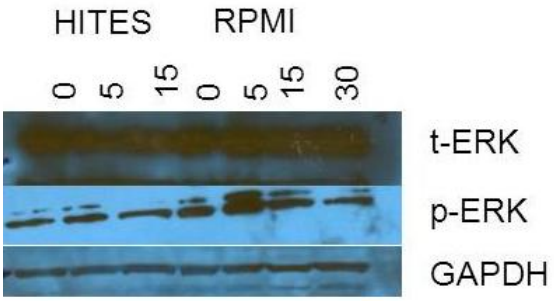


Fig. 12 Western Blot of ERK in NCI-H69 for Medium. Western blotting analysis shows the p-ERK, t-ERK, and GAPDH levels in NCI-H69 cells using 0, 5, 15 and 0, 5, 15 minutes of SDF-1 stimulation in either HITES or RPMI complete medium. The density of t-ERK contained no detectable differences amount the time points. The p-ERK 1/2 levels (1 is the top, 2 is the bottom) increased at 5 minutes for both mediums used. However, there was a greater expression using the RPMI complete medium. The GAPDH shows there is equal protein expression in each sample.

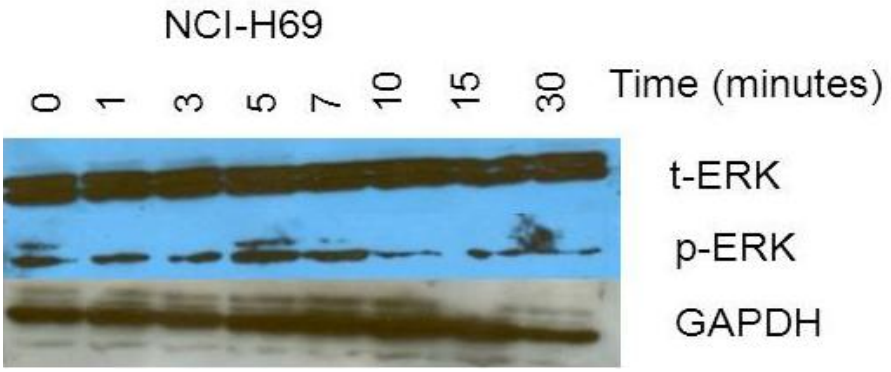


Fig. 13 Western Blot of ERK in NCI-H69. Western blotting analysis shows the p-ERK, t-ERK, and GAPDH levels in NCI-H69 cells using 0, 1, 3, 5, 7, 10, 15, 30 minutes of SDF-1 stimulation. The density of t-ERK contained no detectable differences amount the time points. The p-ERK 1/2 levels (1 is the top, 2 is the bottom) at 5 and 7 minutes, only.

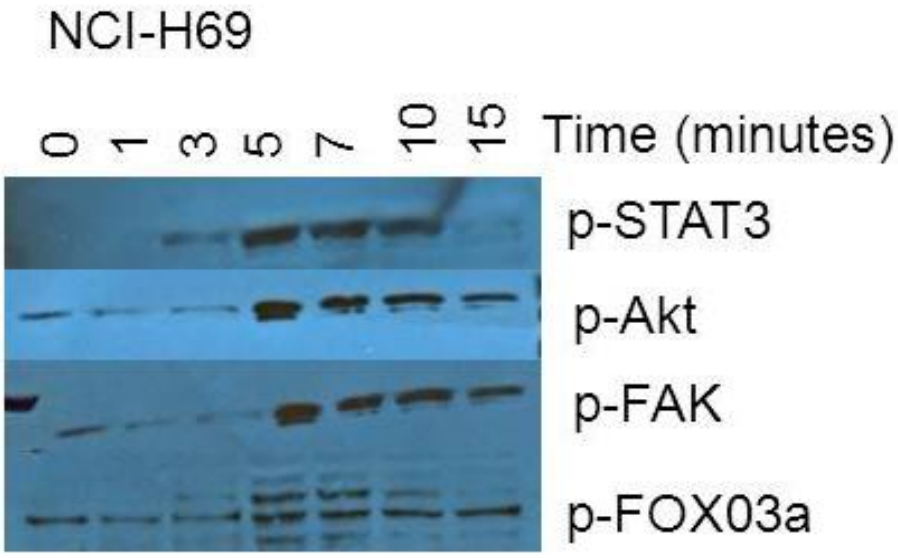


Fig. 14 Western Blot for STAT3, AKT, FAK, and FOX03a in NCI-H69 for Time Points. The Western blot analysis of extracts from NCI-H69 cells, untreated or SDF-1 treated (200 ng/ml) for 0, 1, 3, 5, 7, 10, 15 minutes using p-STAT3, p-Akt, p-FAK, and p-FOX03a antibodies. Expression occurs at 5, 7, and 10 minutes for p-STAT3 and p-FOX03a and at 5, 7, 10, and 15 minutes for p-Akt and p-FAK.

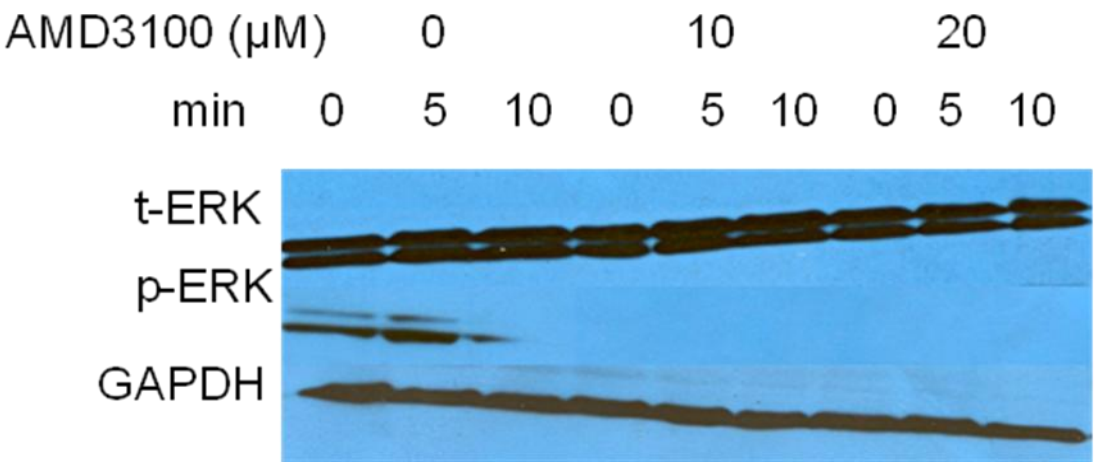


Fig. 15 Effect of AMD3100 on CXCL12 induced ERK Activation in NCI-H69. NCI-H69 cells treated with 0, 10 or 20 μM of AMD3100, and treated with 200 ng/ml of SDF-1 for 0, 5, or 10 minutes. t-ERK and p-ERK primary antibodies used with equal protein expression due to the equal GAPDH expression. There was a decrease in p-ERK expression with increased AMD3100 concentration, which is evident by lack of bands with increasing AMD3100 concentration.

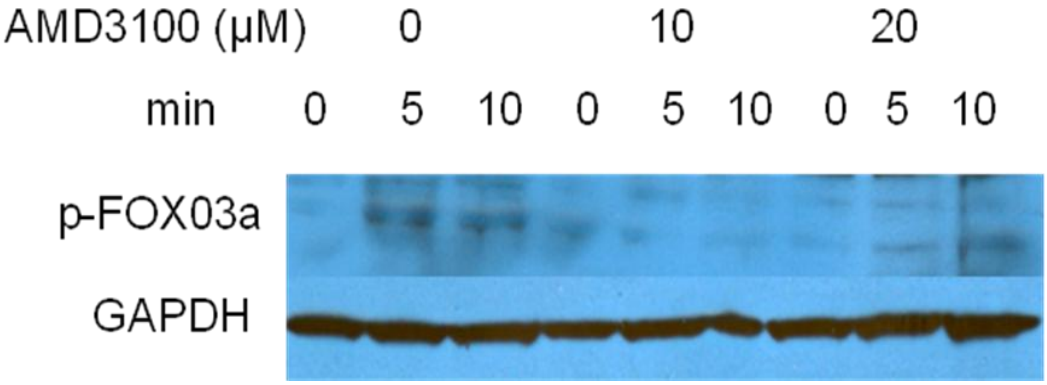


Fig. 16 Effect of AMD3100 on CXCL12 induced FOX03a Activation in NCI-H69. NCI-H69 cells treated with 0, 10 or 20 μM of AMD3100, and treated with 200 ng/ml of SDF-1 for 0, 5, or 10 minutes. p-FOX03a primary antibodies used with equal protein expression due to the equal GAPDH expression. There was a decrease in p-FOX03a expression with increased AMD3100 concentration, which is seen by the lightening of the bands with increasing AMD3100 concentration.

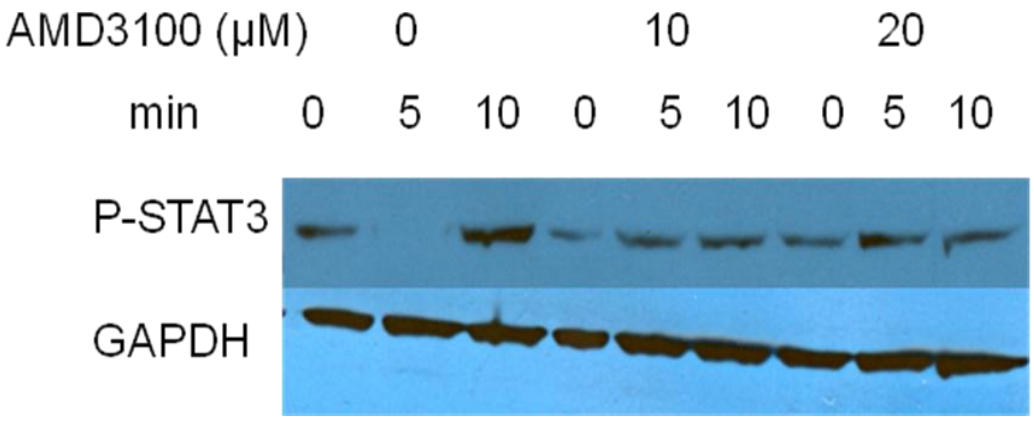


Fig. 17 Effect of AMD3100 on CXCL12 induced STAT3 Activation in NCI-H69. NCI-H69 cells treated with 0, 10 or 20 μM of AMD3100, and treated with 200 ng/ml of SDF-1 for 0, 5, or 10 minutes. p-STAT3 primary antibodies used with equal protein expression due to the equal GAPDH expression. There was a decrease in p-STAT3 expression with increased AMD3100 concentration, which is seen by the lightening of the bands with increasing AMD3100 concentration.

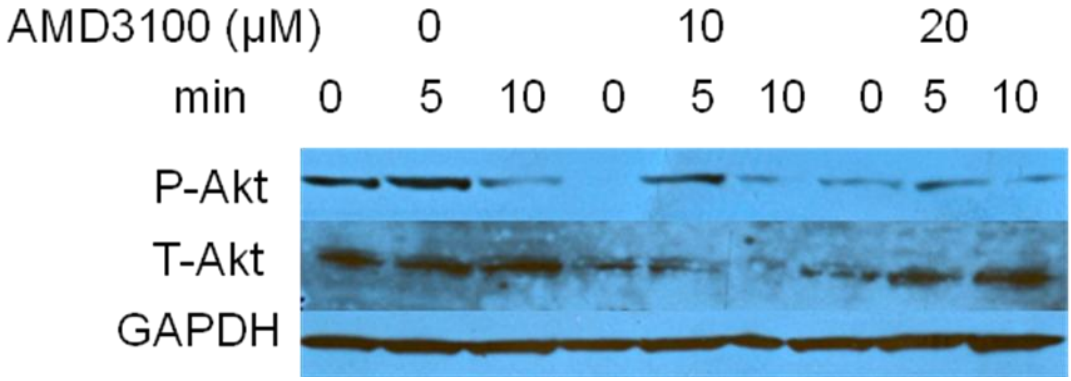


Fig. 18 Effect of AMD3100 on CXCL12 induced Akt Activation in NCI-H69. NCI-H69 cells treated with 0, 10 or 20 μM of AMD3100, and treated with 200 ng/ml of SDF-1 for 0, 5, or 10 minutes. t-Akt and p-Akt primary antibodies used with equal protein expression due to the equal GAPDH expression. There was a decrease in p-Akt expression with increased AMD3100 concentration, which is seen by the lightening of the bands with increasing AMD3100 concentration. The t-Akt bands have relatively the same density throughout the blot.

Chemotaxis and Chemoinvasion Assays

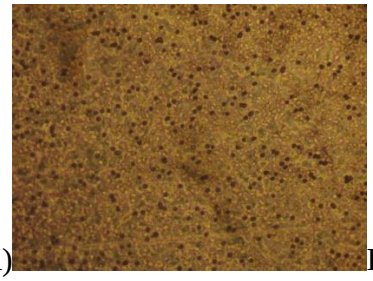
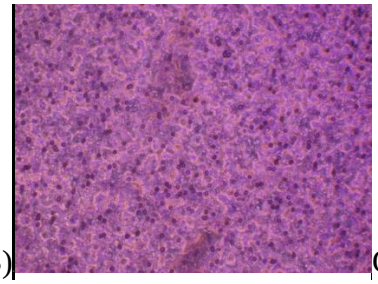
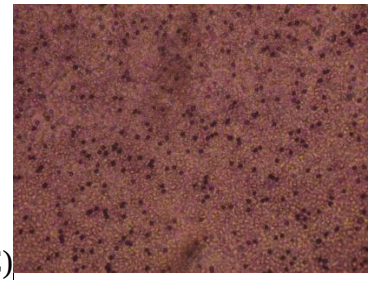
A)  B)  C) 

Fig 7 Cell Staining for the Fourth Migration Assay. SDF-1 ng/ml A) 0. B) 200. C) 400.

In the first migration assay, after three hours, the bottom chamber was checked for cell migration, though there was none. The number of Jurkat cells in the bottom chamber were counted at 24 hours and the H69 cells at 48 hours. The top chambers weren't dyed and discarded. There were 0 cells in the top chambers containing Jurkat cells and an average of 17.5k and 22.5k cells in the bottom chamber for 0 and 100 ng ml^{-1}, respectively. There were no cells in the top chamber of the NCI-H69 cells and 0, 36k, and 28k cells in the bottom chambers for 0, 100, and 200 ng ml^{-1} SDF-1. The wells containing 50 ng ml^{-1} weren't counted due to the low number of cells. The cells bunched together tightly at the membrane, making it impossible to count the number of cells after staining. Trydan blue was used to count the live cells when determining the cell count of a cell solution.

In the Second migration the number of cells in the bottom chamber were counted to be similar in all aspects. The side containing no fibronectin had an average of 21.25k, 40k, and 41.25k cells after 5 hours and 13.75k, 15k, and 18.75k cells after 48 hours for 0, 100, and 200 ng ml^{-1} of SDF-1, respectively. The side with fibronectin coated chambers had an average of 48.75k, 52.5k, and 53.75k cells after 5 hours and 20k, 22.5k, and 26.25k cells after 48 hours for 0, 100, and 200 ng ml^{-1} of SDF-1, respectively. The chambers were not stained.

In the third migration No live cells were found in the top or bottom chamber of the cells, only dead ones. The membranes on chambers were stained after the targeted time was met. Picture were taken, though the clotting effect experienced earlier made it difficult to count the number of cells.

In the fourth No cells migrated past the collagen membrane, but there were cells in the top chambers. The side containing no collagen had an average of 1.5k, 6k, and 6k cells, while the side with collagen coated chambers had an average of 6k, 9k, and 3k cells for 0, 200, and 400 ng ml^{-1} of SDF-1, respectively. The pictures taken had to be zoomed in all the way and the cells didn't possess the typical cell structure seen from a cell caught in a membrane during a migration assay.

In the Fifth migration assay, the results were the exact same as the fourth migration. There is no difference between the 24 hour and 48 hour groups upon observations and pictures obtained. There were not any cells present below the chambers coated with collagen in this assay as well.

Though numerous migration assays were run, the results were inconclusive. No empirical migration data was able to be collected through the difficulties ensued through the congregational trait of the NCI-H69 cell line to bunch together in clusters.

Discussion

Expression of CXCR4 and CXCR7

Jurkat cells were used as a positive control for CXCR4 protein expression using FACS analysis. Their high level of expression supported proper execution of experimental procedure. The assay

run for NCI-H69 showed a large expression of CXCR4 as well. This is important, as CXCR4 is expected to play a large role in metastasis of the NCI-H69 cells with SDF-1 stimulation. If the expression was low, this may not be the case, with little activation occur upon SDF-1 stimulation due to the lack of protein presence. There was a low expression of CXCR4 for the NSCLC cell line A549, which has been shown previous to possess high levels of CXCR4. The procedure may have been run improperly or there may have been a lack of cells to produce such results. New experiments would be run to confirm this if this cell line in future experiments if the research is focused on NSCLC cell lines. There was also a small expression for CXCR7 for NCI-H69 cells. This is contradictory to results from [39] and needs to be run again. CXCR7 is also a receptor for SDF-1, but is believed to modulate CXCR4 instead of directly affecting the metastatic behavior of the cells.

Western Blot

Western blots were run to prove the existence of the CXCR4 protein in the NCI-H69, A549, and Jurkat (positive control) cell lines. A GAPDH assay was run to check the levels of protein expression. They were similar, providing CXCR4's existence in each cell line to be similar and reproducible.

Next, Western blot assays for p-ERK and t-ERK were taken for NCI-H69 cells in order to detect endogenous levels of p44 and p42 MAP Kinase (Erk1 and Erk2) when phosphorylated. Activation of these proteins can show cellular programas such as cell proliferation, differentiation, motility, and death, responding to a diverse range of extracellular stimuli. The results using HITES and RPMI medium show that the RPMI medium give a greater protein

expression due to a healthier cell environment. Also, p-Erk2 is expressed in a greater quantity around 5 and 10 minutes than 0 or 15 minutes. Upon further testing, the greatest expression is at 5 and 7 minutes. Upon further testing using SDF-1 as stimulation and testing the protein expressions of p-FAK, p-FOX03a, p-Akt, and p-STAT3, optimal results were obtained at 5 and 10 minutes. This concluded that 0, 5 and 10 minute stimulations using SDF-1 would be used for further western blots with several variables.

p-FAK, p-FOX03a, p-Akt, and p-STAT3 are all important in monitoring cell metastasis. The protein p-Akt is important in regulation cell survival. An increase in p-Akt can show that cells have oncogenic properties. The protein p-FAK is important in focal adhesion kinases and it plays a role in cell migration, and shows that cells are in a migratory phase if there is an increased expression. The protein p-STAT3 is important in pro-inflammatory pathways which has been shown to enhance tumor growth and metastasis, with increase levels showing tumorigenic properties. Also, the protein p-FOX03a is of extreme interest in relation to develop drug resistance to chemotherapy, with increased levels pointing at more aggressive tumorigenic cell properties.

The Western blots for H69 treated with the AMD3100 showed expected results when treated withp-FOX03a, p-Akt, T-Akt, p-STAT3, p-ERK, and T-ERk, and are all important in monitoring cell metastasis. The decrease in protein levels when stained with p-Akt with increased levels of AMD3100 used shows that the CXCR4 antagonist help in preventing oncogenic properties. The protein staining for p-FAK didn't show up well, which is most likely attributed to the primary solution going bad. The decreased protein levels when treated with p-STAT3 resulted in a decrease in pro-inflammatory pathways, decreasing tumor growth and metastasis when increasing concentrations of AMD3100 was used. Also, the decreased protein levels of p-

FOX03a represents a decrease in aggressive tumorigenic cell properties as a higher concentration of AMD3100 was used.

Chemotaxis and Chemoinvasion Assays

None of the migration assays seemed to be successful, even after repeating the same procedures as listed in [27], which were listed with observable results. The cells all either died, disintegrated, or clogged the membrane coating of the chambers. The cells could have died from the lack of FBS used and the length of time they were incubated, though results were achieved using similar procedures, as state in [27]. Also, small amounts of FBS were added in later trials, which didn't solve this issue. The cells also congregate into bunches which may have plugged up the chamber pores, making it hard to count the cells that were stuck in the chamber membrane or allowing them to pass through the membrane. The few migrations that seemed initially to have worked may have only been a staining of the actual pores, without cell existence. These stainings lacked a nucleus. Several migration assays have been run for NCI-H69, with none have been done for NCI-H345, which congregate and will most likely contain the same issues. According to [27], NCI-H69 and NCI-H345 cells both migrate with SDF-1 stimulation in vitro, though my results were inconclusive.

Conclusion

We established that SCLC express chemokine receptors CXCR4 and CXCR7 through western blot and FACs analysis. That CXCL12 ,the ligand for these receptor, was shown to induce

signaling in SCLC cells as detected by enhanced phosphorylation of ERK, Akt, FOX03a, and STAT3 through western blots analysis in induced cells. Furthermore, AMD3100, the inhibitor for CXCR4, showed a decrease of CXCL12 induced ERK, Akt, FOX03a, and STAT3 phosphorylation. These results suggest that CXCL12, upon binding to CXCR4, may induce signaling mechanism that may enhance proliferation, chemotaxis, tumor growth, and metastasis in SCLC cells. In addition, AMD3100, which blocks the CXCR4 mediated functional effects and signaling, could be used as a therapeutic strategy to inhibit small cell lung cancer, which has limited therapeutic options.

Future Research

Therefore, in our study, we will use a detailed analysis of CXCR4/CXCR7 expression in a wide spectrum of lung cancer tissue microarray (TMA) patient samples, including SCLC, and correlate their expression with clinical pathological findings. The next step would be to determine the effect of CXCR4/CXCR7 inhibitors on tumor growth and metastasis in *in vivo* mouse models using SCLC cell lines. Furthermore, we will determine the effect of cisplatin on generating CD133/CXCR4+ stem-cell like populations, which may be responsible for developing drug resistance. We will also determine if CXCR4 inhibitors could be used to inhibit growth of drug-resistant SCLC cells. Further understanding of expression of CXCR4/CXCR7 in SCLC, and *in vivo* pre-clinical studies with CXCR4/CXCR7 inhibitors, may provide a rationale for developing CXCR4/CXCR7 as potential novel therapeutic targets in SCLC.

Acknowledgements

I would like to thank Dr. Ramesch Ganju, Dr. Mohd Nasser, Dr. Dinesh Ahirwar, Dr.Nissar Wani, Catherine Powell, Gary Helong Zhou, and Nagaraja Tirumuru for advising, as well as training me during this research project. Thank you to the Pelotonia fellowship for this amazing research opportunity and all the people that donated towards the cure for cancer. I would also like to thank Merijn van der Heijdan and the Arts and Sciences Honors Program for approving and supporting my thesis to graduate with research distinction. I would also like to thank Dr. Xue-Feng Bai for being the Pathology departmental representative during my oral defense of my thesis. And lastly, I would like to thank my parents, Janet and Bill Brumfield for helping me achieve my goal of graduating college.

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